Waves of mouse *Lunatic fringe* expression, in four-hour cycles at two-hour intervals, precede somite boundary formation

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During somitogenesis, cells are recruited to the caudal presomitic mesoderm (PSM) from the primitive streak (and later the tail bud), while somites separate from the rostral end as epithelial cubes [1]. This is a regular process, one somite forming every 2 hours in the mouse, that can be simulated by clock and wavefront models [2]. The chick basic helix-loop-helix transcription factor encoded by *c-hairy1* is expressed in dynamic waves in the PSM, undergoing one cycle for each somite formed [3]. This is compatible with an underlying oscillating molecular clock. We have shown here that *Lunatic fringe* (*L-fng*) expression is indicative of it being one of the implementing outputs of this clock. *Fringe* genes regulate the Notch signalling pathway in boundary formation [4,5]. Of the known mouse genes, only *L-fng* is expressed in PSM [4,5] and it is required for somite segmentation and patterning [6,7]. We have now shown that *L-fng* is expressed as dynamic, repetitive and complex waves within the mouse PSM. A wave takes 4 hours to complete one cycle and terminates immediately at, and prior to, somite boundary formation. Consecutive waves are temporally but not spatially overlapping, being initiated in the caudal PSM every 2 hours, so offset by one half-cycle. Waves of expression are not associated with cell movement and do not require cell contact for propagation, so appear to reflect a cell-autonomous clock that is synchronous in all PSM cells.

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Received: **17 July 1998**
Revised: **11 August 1998**
Accepted: **12 August 1998**

Published: **31 August 1998**

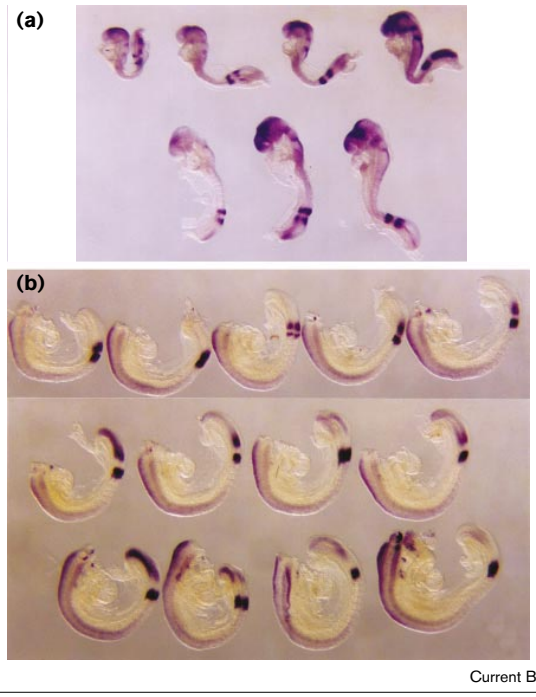
Current Biology 1998, 8:1027–1030
http://biomednet.com/elecref/0960982200801027

Results and discussion

Throughout the period of somite formation in the mouse (embryonic days E7.5–E11.5), we found that *L-fng* expression appears as one, two or three bands within the PSM, largely as previously observed [4,5]. Even in a group of embryos with the same number of formed somites, *L-fng* expression in the PSM appears equally variable (Figure 1). A detailed examination reveals that *L-fng* is never expressed in a formed somite; bands in the rostral PSM are always narrower than those positioned caudally and, most importantly, there are consistent relationships between the locations and widths of bands in an individual embryo (Figure 2). One possible explanation for these static patterns is that they represent different stages in a rapid cycle of expression.

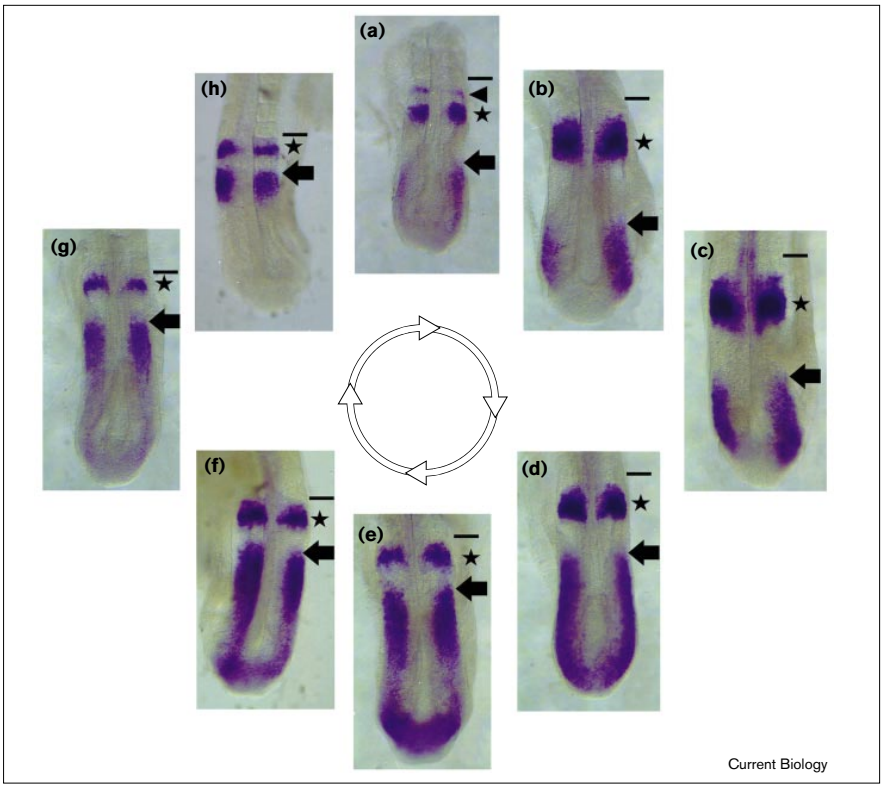
To test this possibility, we bisected along the midline the PSM-containing region of E8.5 embryos, removed and fixed one half, to reveal time-zero expression, and cultured the remaining embryo for 30–120 minutes to evaluate any change in expression. Bands of *L-fng* expression in the cultured halves were always different ($n = 28$) from the contralateral fixed halves (Figure 3). Bands in the rostral PSM

Figure 1



Whole-mount *in situ* hybridisation showing *L-fng* expression in **(a)** E8.5 and **(b)** E9.5 mouse embryos. Most commonly, two bands of expression are apparent in the PSM, of variable rostro-caudal length, but there are also embryos with one or three bands. The group of E9.5 embryos (which have had their heads removed) are of very similar developmental stage (15–18 somites).

Figure 2



Different phases **(a–h)** of a putative temporal and spatial cycle of *L-fng* expression in E9.5 mouse PSM (dorsal views, rostral at top). From a phase in which the caudal half of the PSM lacks expression (h), a new caudal *L-fng* pulse is initiated (a), then strengthens (b) and expands rostrally and caudally (c) to encompass the tail tip (d). The rostral margin of this band is indicated by an arrow. Expression then decreases in the same region as it started (e) and tail tip expression gradually disappears (f–h) as the band condenses while moving rostrally. Rostral to this band is another band (indicated by a star) undergoing further condensation and rostral movement (b–h); a third yet more rostral band, narrowed and fainter, is indicated by an arrowhead in (a). In the cycle, the bands indicated by the arrow and star in (h) become, respectively, the bands indicated by the star and arrowhead in (a). In each panel, the most caudal somite boundary is marked with a horizontal bar.

were usually narrower and shifted rostrally, compared with the corresponding band at time zero. These shifts were progressive with longer periods of culture until, by 120 minutes, the expression patterns of the embryo and its isolated fragment appeared similar once again (Figure 3).

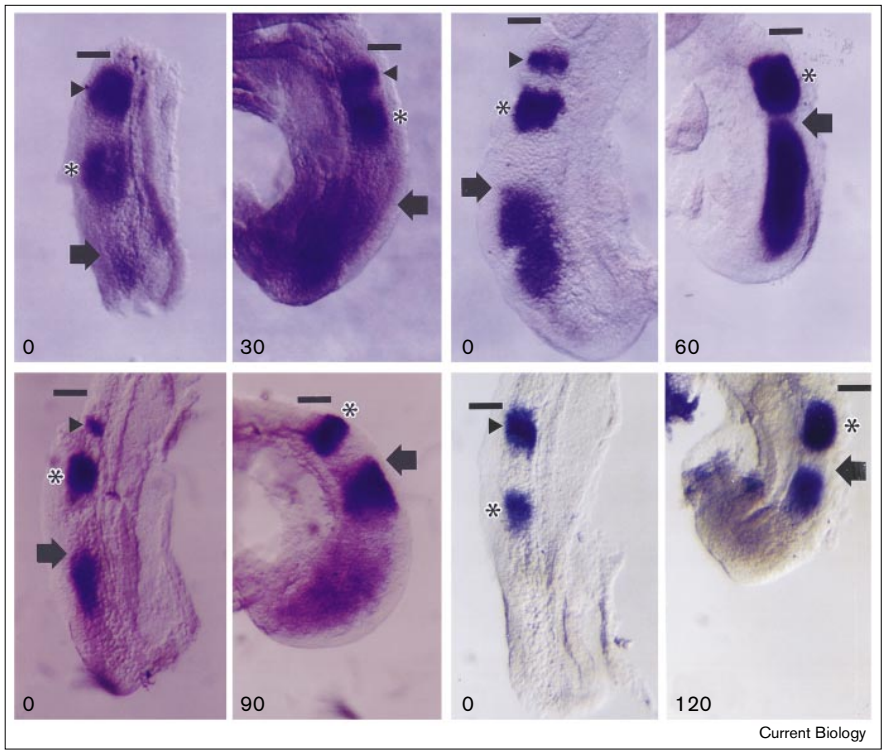
Taking the static and dynamic expression analyses together, a picture emerges of travelling domains of *L-fng* expression that last about 4 hours, initiated every 2 hours, as depicted in Figures 2 and 4. The wave appears in the caudal PSM, spreads throughout the tail bud, and rostrally to a sharp margin. Downregulation, caudo-rostrally, leaves a broad band which narrows, then disappears, before a somite boundary forms, just caudal to it.

Three facts are noteworthy from this analysis. First, we cannot judge accurately the lengths of individual portions of the cycle, so we make no claim that the diagrams in Figure 4 are evenly spaced in time. Second, there can be slight asynchrony between consecutive waves; a wave can be initiated either just before or just after the disappearance of the wave two cycles before. Third, we have never seen spatially overlapping waves, that is, there is always a distinct region of nonexpression between bands.

To examine the possibility that the waves of expression reflect underlying movement of cells, we labelled groups of PSM cells in intact E8.5 embryos with the fluorescent carbocyanine dye DiI, then followed their location after 1, 2 or 3 hours in culture. At this stage, a new somite is about 100 μm in length and the PSM about 900 μm. We did not observe any rapid large-scale movement of cells. A bolus of dye placed 300–500 μm from the caudal end of the PSM was steadily displaced rostrally by approximately 20 μm per hour, relative to the caudal tip of the PSM (n = 16 injections). Thus, the waves of *L-fng* do not correspond to a particular population of cells, but must reflect the coordinated up- and down-regulation of expression.

L-fng expression could be controlled from an initiating point source, perhaps within the caudal/lateral part of the PSM, where the wave appears to start. This could be attained by a diffusing signal molecule, or propagated by a relay mechanism. To test this, we removed the caudal half of the left or right PSM, cultured the remaining embryo for 1 hour, then examined *L-fng* expression. In all cases (n = 16), rostral *L-fng* expression on the side without caudal PSM was identical to the intact side (data not shown). This rules out a controlling signal from caudal PSM, whatever the mode of propagation. Thus, it appears that the fluctuations in *L-fng* expression within the PSM are cell autonomous, reflecting some underlying molecular clock, and, further, this clock is synchronous throughout

Figure 3



Progression of *L-fng* expression over a 30–120 min time period (lateral views, rostral to top). In each pair, the isolated half of the PSM (0 min) is marked with the same symbols as in Figure 2 and is compared with its contralateral half that remained intact within the E8.5 embryo during growth in culture, for which the symbols are consistent with the time 0 panel. After 30 min, the two rostral bands (arrowhead and star) have narrowed and moved rostrally, and the faint caudal band (arrow) has strengthened and expanded (as in Figure 2h/a to a/b or Figure 4 just before and after step A). After 60 min, the most rostral band (arrowhead) has disappeared and the caudal band (arrow) has expanded, strengthened and moved rostrally (as in Figure 2a to c/d or Figure 4 steps A to B/C). After 90 min, the most rostral band (arrowhead) has disappeared, the second and third bands (star and arrow) have progressed rostrally and a new caudal band is emerging (as in Figure 2a/b to h/a or Figure 4 steps A/B to E/A). After 120 min, the pattern appears the same, but the rostral band (arrowhead) at time 0 has been replaced by the second band (star) and a new second band (arrow) has developed (as in Figure 2h to h, or Figure 4 steps 1-E to 2-E).

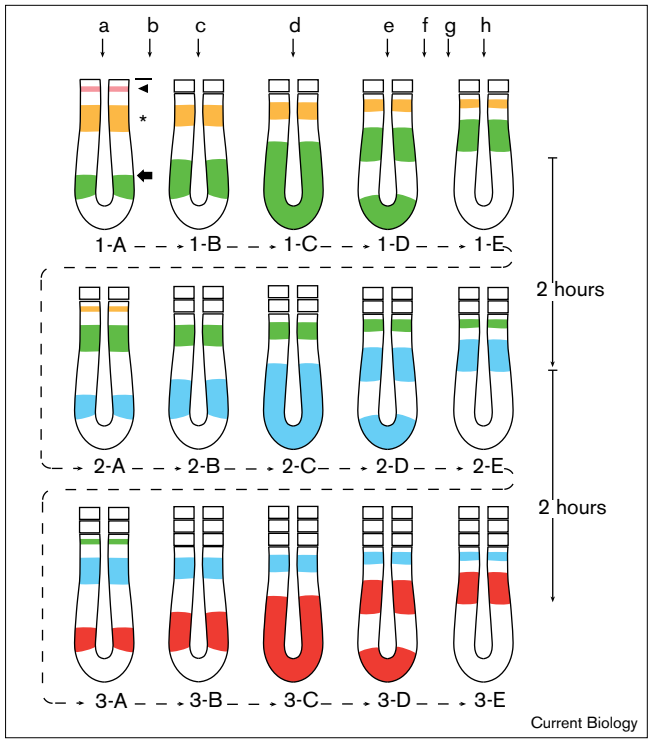
the PSM, suggesting an earlier 'setting', perhaps in the primitive streak.

Many aspects of mouse *L-fng* expression are very similar to expression of the chick transcription factor gene *c-hairy1* [3]; in particular, the periodicity in both cases corresponds to the formation of one somite (every 90 minutes for chick and every 2 hours for mouse). The mammalian homologue of *c-hairy1* has yet to be identified, but may lie upstream of *L-fng*. Previous reports of chick *L-fng* expression did not describe waves in the PSM [8], but in a recent study (published in *Current Biology* while this article was in press), McGrew *et al.* [9] have now shown very similar dynamics in chick as we have observed in mouse. They also report synchrony of *c-hairy1* and *L-fng* expression during the caudal portion of their waves. There are subtle spatial differences between the apparent waves of *L-fng* in mouse and chick, and expression remains in the anterior of just-formed somites in chick but not mouse. These comparisons give no clue, however, to the function of the *L-fng* waves, and there is no precedent for rapid oscillation in an mRNA that encodes a secreted protein.

In *Drosophila*, Fringe inhibits Serrate and potentiates Delta, and a feedback loop restricts Notch activation to a border region [10]. The exact mechanism of Fringe activity is unknown, although structural similarity with bacterial glycosyl transferases [11] suggests modulation of the carbohydrate moieties of membrane-bound glycoproteins. Although Fringe has the pre-pro sequences typical of secreted proteins, it has been suggested to act cell autonomously and to associate with the cell membrane [10]. In the mouse, several genes encoding other members of the Notch pathway, in addition to *L-fng* [6,7], are required for somitogenesis [12,13]. Previous studies give no evidence for dynamic or cyclic expression of known Notch receptors or ligands in mouse PSM [12–15], and we have confirmed these observations (data not shown). The transcription factor encoded by *Hes-5*, however, which was earlier thought to be specific to the central nervous system [16], may act downstream of *L-fng*. Limited data show an intriguing *Hes-5* expression pattern in the mouse PSM [14], and other *Enhancer-of-split*-like genes are activated by mammalian Notch [17].

As groups of cells destined to form a somite traverse the PSM, they will undergo a series of *L-fng* expression pulses of less than 2 hours, perhaps 8 hours or so in total, followed by a longer final pulse. Assuming that the protein is stable, *L-fng* mRNA pulses would sequentially increase, in ratchet fashion, the extracellular/membrane levels of L-Fng protein, which would become proportional to the number of transcription cycles experienced. The formation of a new intersomite boundary could then be triggered at a threshold level of L-Fng. A switch from Serrate to Delta signalling, or restriction of Notch activation,

Figure 4



Diagrammatic model of the waves of *L-fng* expression during the development of three somites. One complete wave of expression (green) is illustrated in steps 1-A to 3-A, along with the concurrent two preceding (pink and orange) and subsequent (blue and red) waves. A somite boundary forms just after the end of a wave, and just caudal to it, as in steps A–B. The letters a–h at top indicate the stages of *L-fng* expression shown in Figure 2a–h and the symbols (arrow, star, arrowhead and bar) in 1-A are as defined in Figure 2.

could possibly allow the formation of a boundary and separation of the epithelial somite from PSM. Probably the greatest challenge of these observations is to understand the control of *L-fng* transcription, and thus the remarkable synchronous clock entrained in the PSM.

Materials and methods

Animals and in situ *hybridisation*

F2 embryos from B6CBF1 mice were used throughout. Whole-mount *in situ* hybridisation was performed essentially as described by Henrique *et al.* [18], using a 759 bp probe (nucleotides 390–1149 from start site) representing most of the mature protein [4,5].

Bisection and transection of PSM

The visceral yolk sac of E8.5 conceptuses was slit in the avascular perichorionic region, the caudal end of the embryo being exteriorised. In bisection experiments, the caudal embryo was cut through the neural midline and one half (left or right), including the whole PSM and one or two somites, was cut from the embryo and immediately fixed in 4% paraformaldehyde in phosphate-buffered saline. In transection experiments, the midline cut was made to about half way up the PSM then the left or right caudal end of the embryo, including half the PSM on that side, was removed. In both types of experiment, the remaining embryo, still attached to yolk sac and amnion, was cultured for 30–120 min in minimal essential medium (MEM) containing 50% rat serum at 37°C, in 5% CO_2, then fixed. Embryos and their matching presomitic fragments were then processed together for *in situ* hybridisation.

DiI labelling of PSM cells

Groups of cells were labelled by microinjecting a bolus of the dye DiI (1,1′-didodecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate) into the PSM of intact E8.5 conceptuses (10–12-somite stage). The location of the dye relative to the caudal end of the embryo was recorded, conceptuses cultured for 1, 2 or 3 h in 75% rat serum, 25% MEM in roller bottles, then the location re-recorded.

Acknowledgements

We are grateful to Ian Andrew for his technical skill, Tim King for advice, Sean Egan for *L-fng* cDNA, the US EPA for a cooperative agreement and the UK MRC for additional funding.

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